



Clustered Carbohydrates as a Target for Natural Killer Cells: A Model System



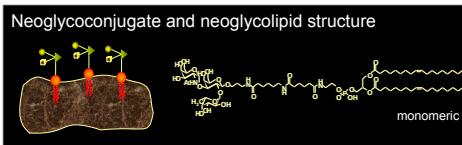
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Abstract

Functional activity of NK cells, a critical component of the innate immune system, is dependent on oligosaccharides for successful receptor-ligand interactions. Synthetic glycoconjugates are an important tool for studying these interactions; we therefore used monomeric, polymeric and lipophilic forms of these molecules to study the role of carbohydrate involvement in NK cell cytotoxicity and cytokine production. Target cells (K562) were modified by incorporation of lipophilic glycoconjugates containing different saccharides, making carbohydrate residues available for NK-target cell interactions. A broad panel of biologically important saccharides were tested, included blood group antigens, ligands of selectins, tumor-associated antigens and xenobiotics. Incorporation of neoglycoconjugates into the cells was initially measured using fluorochrome-conjugated oligosaccharides analyzed by flow cytometry. Saccharides that successfully bound to target cells and remained on the cell surface for extended times were subsequently assessed for their ability to modulate NK-mediated cytotoxicity, using a fluorogenic caspase 6 substrate, and IFN-g production by ELISA. CD3-CD16+CD56+ and CD3-CD16-CD56+ purified NK cell subsets were used, isolated both by magnetic separation and by FACS. Most of glycoconjugates did not result in any change in NK cytotoxicity. However modification of target cells with polymeric glycoconjugates containing Le^x, HS03Le^x and Le^y sharing the common structure motif trisaccharide Lex evoked an increase of NK-mediated lysis of the cells. In contrast, monomeric Le^x, HS03Le^x and Le^y containing glycoconjugates did not affect cytotoxicity. Lipophilic polymeric and monomeric glycoconjugates as well as free saccharides had no effect on IFN-g production. Only non-lipophilic polymeric glycoconjugates containing indicated saccharide determinants evoked moderate IFN-g production by NK cells. The observed stimulating effect of glycoconjugates was found to be connected mainly with the CD3-CD16+CD56+ cell subset. Thus, the glycoconjugate effects were dependent on saccharide presentation in polymeric or clustered form, suggesting that appropriate presentation is critical for carbohydrate recognition and subsequent biological effects.



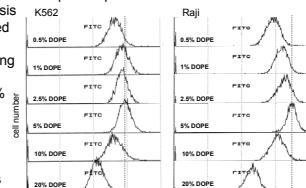
Incorporation of polymeric neoglycoconjugates into cells and detection by flow cytometry



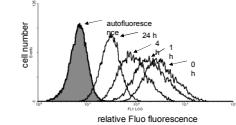
Oligosaccharides are critical structural and functional components of glycoproteins, necessary for successful receptor-ligand interactions. **Neoglycoconjugates** (synthetic polymeric constructs that incorporate saccharide molecules) are important tools for studying these interactions; they can selectively affect glycoprotein-dependent biological activities when incorporated into cells. In this study, we have developed flow cytometric methodology to monitor the incorporation of fluorochrome-conjugated synthetic glycoconjugates into cells, and used this system to study the effects of these molecules on NK-mediated target cell cytotoxicity.

The lipophilic FITC-conjugated glycoconjugate Le^x-PAA(Fluo)-DOPE(5%) was incubated with K562 and Raji cells over time and analyzed by fluorescence microscopy (*top photo*) and flow cytometry (*lower plot*). Good incorporation could be detected by 45 minutes by 37°C, and could be monitored until incorporation reached a steady state.

A hydrophobic lipid anchor (DOPE) was incorporated into these synthetic glycoconjugates to improve uptake and retention.



Retention and turnover dynamics of glycoconjugate incorporation were then measured by incubating K562 target cells with Lex-PAA(Fluo)-DOPE(5%) and analyzing glycoconjugate fluorescence at timepoints over a 24 hour period (below). Retention was stable for up to 4 hours, and glycoconjugate was easily detectable even after 24 hours.

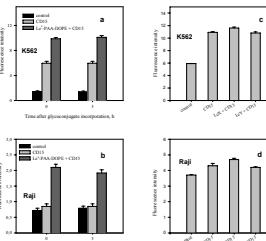


Saccharides available

Bdi: Gal β 1-3Gal β ,
Btri: Gal β 1-3(Fuc α 1-2)Gal β ,
Lex: Gal β 1-4(Fuc α 1-3)GlcNAc β ,
Ley: Fuc α 1-2Gal β 1-4(Fuc α 1-3)GlcNAc β ,
HS03Lex: HS03-3Gal β 1-4(Fuc α 1-3)GlcNAc β ,
HS03Lea: HS03-3Gal β 1-3(Fuc α 1-4)GlcNAc β ,
SiaLex: Neu5Ac α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc β ,
Sia2: Neu5Ac α 2-Neu5Ac α ,
3'SL: Neu5Ac α 2-3Gal β 1-4Glc β ,
Tbb: Gal β 1-3GalNAc β ,
Tr: GalNAc-,
SiaTn: Neu5Ac α 2-6GalNAc α ,
asialoGM1: Gal β 1-3GalNAc β 1-4Gal β 1-4Glc β .

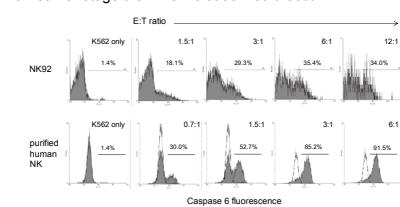
Do neoglycoconjugates make recognizable epitopes?

Although synthetic glycoconjugates do associate with cells, it is not clear whether they possess functionality by this association. To show **functional incorporation of glycoconjugates**, K562 and Raji cells were immunolabeled for CD15 (the epitope being a Le^x trisaccharide) after multimeric Le^x-PAA(Fluo)-DOPE(5%) incorporation. *Graphs A and B* below demonstrate that Lex-PAA(Fluo)-DOPE(5%) could be detected on the surface of K562 and Raji cells by fluorescent immunolabeling with anti-CD15 antibodies. This synthetic incorporation was therefore recognizable as a cell surface epitope. Interestingly, monomeric Le^x did not significantly affect anti-CD15 labeling (shown in graphs C and D), suggesting that multimeric presentation was important for epitope recognition on the cell surface.



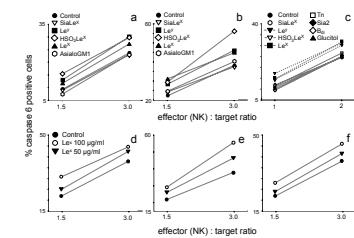
NK-mediated cytotoxicity by flow cytometry

These synthetic glycoconjugates were then used to study the role of oligosaccharides in a real biological event, namely **natural killer (NK) cell recognition and lysis of tumor target cells**. Caspase 6 activation was used as a flow cytometric assay for NK cytotoxicity, using a fluorogenic caspase 6 substrate from Oncorimmunin, Inc. (Gaithersburg, MD, USA). An example is *below*, using both NK92 and purified human NK cells with a K562 target, at different K:T ratios. Target cells were prelabeled with the caspase substrate and a fluorescent tracking dye to distinguish them from NK cells. Early NK-mediated cytotoxicity was readily detected by flow, at an earlier stage than ⁵¹Cr release would occur.

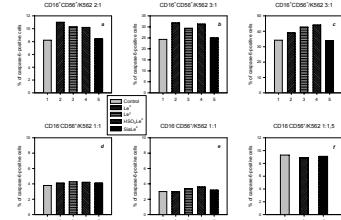


Can neoglycoconjugates module NK-mediated cytosis of tumor target cells?

A large panel of synthetic glycoconjugates was then analyzed for their effect on **purified human NK cell mediated cytosis of a K562 target cell line**. NK cells were obtained from whole blood, and purified for CD3-CD16+CD56+ cells by magnetic separation to > 85%. Neoglycoconjugates were incorporated into target cells and co-incubated with NK cells. Polymeric Le^x and Le^y glycoconjugates increased NK-mediated cytosis, while other glycoconjugates did not exert significant effects. The *upper row* of plots shows five representative glycoconjugates, while the *lower row* shows three repeat experiments using Le^x and Le^y. Polymeric Le^x and Le^y also enhanced IFN γ production by NK cells, also important for NK activity (data not shown). Monomeric Le^x and Le^y showed no effect, again showing the importance of a multimeric recognition site.



Human NK cells were then separated into the CD3-CD16-CD56+ fraction (about 10% of normal NK cells, with less cytolytic activity than CD16+ cells), and the remaining CD3-CD16+CD56+ fraction by fluorescence activated cell sorting. The enhancement of K562 target cytosis by Le^x and Le^y containing glycoconjugates was restricted to the CD3-CD16+CD56+ fraction (top row, three separate experiments). The CD3-CD16-CD56+ fraction showed by effect of glycoconjugates (lower row, three separate experiments).



Synthetic glycoconjugates can be designed that mimic the biological activities of native oligosaccharides. Polymeric glycoconjugates appear to have more biological activity than monomeric constructs.

These glycoconjugates can be coupled to fluorescent molecules and monitored for uptake and retention in cells by flow cytometry.

These glycoconjugates can form recognizable epitopes on the surface of cells, and can modulate important receptor-ligand recognition events (such as NK-mediated target cell cytosis).